

**PATENT**  
**ARCD:343US**

**APPLICATION FOR UNITED STATES LETTERS PATENT**  
**for**  
**METHOD FOR GENERATION OF LONGER cDNA FRAGMENTS**  
**FROM SAGE TAGS FOR GENE IDENTIFICATION**

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## **BACKGROUND OF THE INVENTION**

The present application claims the priority of co-pending U.S. Provisional Patent Applications, Serial No. 60/173,617, filed December 29, 1999, and Serial No. 60/174,391, filed January 3, 2000, the entire disclosures of which are incorporated herein by reference without disclaimer. The government owns rights in the present invention pursuant to grant number CA42557 from National Institutes of Health and CA78862-01 from the National Cancer Institute.

### **1. Field of the Invention**

The present invention relates generally to the field of genome-wide gene analysis. More particularly, it concerns the development of a technique wherein longer sequences extended from SAGE tags are generated to analyze gene expression. Furthermore, it concerns the development of a technique wherein extended DNA sequences encoding parts of an isolated protein fragment are generated to identify genes encoding isolated proteins. The invention also provides a high-throughput method for identifying genes encoded by SAGE tags.

### **2. Description of Related Art**

A particular biological event in a cell is largely controlled by the expression of multiple genes, both at the correct time and in a spatially appropriate manner. Monitoring the pattern of gene expression under various physiological and pathological conditions is a critical step in understanding these biological processes and for potential intervention. Because of the large number of genes expressed in higher eukaryotic genomes, powerful tools are needed to characterize the overall pattern of gene expression. The successful development of the SAGE technique (Serial Analysis of Gene Expression) is an important milestone in this regard (Velculescu *et al.*, 1995). In the SAGE technique, a short sequence tag with 10 base nucleotides representing each expressed sequence is excised and the tags from different expressed sequences are ligated for sequencing analysis. This strategy provides maximal coverage of the expressed genes for gene identification at the whole genome level while keeping the sequencing analysis

at a manageable scale. Application of the SAGE technique has provided valuable information in various biological systems (Zhang *et al.*, 1997, Velculescu *et al.*, 1997, Madden *et al.*, 1997, Hibi *et al.*, 1998, Hashimoto *et al.*, 1999).

5           However, there are two problems when applying the SAGE tag sequence for gene identification. The first is that many SAGE tags identified have no match to known sequences in databases (Zhang *et al.*, 1997, Velculescu *et al.*, 1997). These tags may represent potentially novel genes. It is difficult, however, to use this tag information for further characterization of the corresponding genes because of their short length. The  
10           second problem is that many SAGE tag sequences have multiple matches with sequences in the databases. These matched sequences have no similarity to each other except that they share the same SAGE tag sequence. This feature makes it difficult to determine the correct sequence in a particular tissue corresponding to a SAGE tag among these matched sequences.

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### SUMMARY OF THE INVENTION

20           To overcome these problems, the present inventors developed a technique called the Generation of Longer cDNA fragments from SAGE Tags for Gene Identification (GLGI). The key features of this technique are the use of a sequence containing a SAGE tag as the sense primer, and the use of a single-base anchored oligo-dT as the antisense primer, and *Pfu* DNA polymerase for PCR amplification. By using this approach, a SAGE tag sequence can be converted immediately into a longer cDNA fragment containing up to several hundred bases from the SAGE tag to the 3' end of the  
25           corresponding cDNA. The development of the GLGI technique overcomes the two obstacles discussed above and should have wide application in SAGE-related techniques for global analysis of gene expression. The same principle can be applied to confirm the reality of genes predicted by bioinformatics tools.

30           Therefore, in one embodiment of the present invention, there is provided a method for characterizing a SAGE tag fragment comprising (a) obtaining a RNA sample

from the same tissue type as used in generating said SAGE tag; (b) generating cDNA fragments that correspond to the SAGE tag from said RNA sample by performing a DNA amplification reaction wherein primers used comprise:

- (i) a SAGE tag sequence as a sense primer; and
  - 5 (ii) at least one single-base anchored oligo-dT primer as an antisense primer; and
  - (iii) analyzing said cDNA fragments. The RNA sample preferably is the RNA sample used to perform SAGE. The DNA amplification preferably comprises polymerase chain reaction, for example, using *Pfu* DNA polymerase. The  $Mg^{2+}$  concentration preferably is 4 mM. The cDNA fragments generated are generally about
- 10 50 to 600 base pairs in length.

The method uses single-base anchored oligo-dT primers comprising a single-base anchored to the 3' end of the oligo-dT primer said base excluding dT, preferably

15 ~~comprising from 10 to 25 poly-dT residues, even more preferably 11 poly-dT residues.~~

The sense primer may further comprise a *Bam*HI recognition sequence at the 5' end. The SAGE tag may further comprise a *Nla*III recognition sequence at the 5' end.

The method may further comprise cloning cDNA fragments, sequencing the clones to identify the cDNA fragment sequence, and comparing the cDNA sequence to

20 sequences in existing DNA databases. Alternatively, the method may comprise hybridizing the cDNA fragments with known sequences. In a more specific embodiment, the method comprises performing a DNA amplification reaction using (a) a sense primer designed based on an existing exon sequence, (b) a single-base anchored oligo-dT primer

25 as an antisense primer, and (c) cloning and sequencing the amplified DNA. Cloning may advantageously include cloning into an expression vector, including a promoter operable in prokaryotic or eukaryotic cells. The exon sequences may be predicted by bioinformatics tools. The amplified sequences may be aligned with genomic DNA sequences.

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The tissue type may be colon, thymus, small intestine, heart, placenta, skeletal muscle, testes, bone marrow, trachea, spinal cord, liver, spleen, brain, lung, ovary, prostate, skin, cornea, retina, and breast.

5           The present invention also describes a method for identifying a gene comprising:  
a) obtaining an isolated protein; b) digesting said protein to obtain at least a first protein  
fragment; c) obtaining at least a first amino acid sequence from said first protein  
fragment; d) generating a first DNA fragment that encodes said first protein fragment; e)  
10 performing a DNA amplification reaction with cDNA obtained from the same tissue  
sample as the isolated protein wherein primers used comprise: (i) a sense primer  
comprising said first DNA; and (ii) at least one single-base anchored oligo-dT primer as  
an antisense primer; and f) analyzing said cDNA fragments.

15           In one embodiment of the method the steps c) through f) are repeated with other  
protein fragments generated by the digestion. For example, the steps c) through f) can be  
repeated with a second protein fragment, a third protein, a fourth protein fragment, or a  
fifth protein fragment to mention a few. In some specific embodiments, at least three  
amino acid sequences are obtained from the protein.

20           In some embodiments of the method digesting the protein is followed by a  
separation to obtain purified protein fragments. The digestion may comprise the use of  
proteases well known in the art such as trypsin, chymotrypsin, elastase, collagenase,  
leupeptin and endopeptidases. Other protein digesting enzymes may also be used.  
Separation of the digested protein fragments may be based on the size of the protein  
25 fragments.

30           In specific embodiment of the method the separation and purification may involve  
protein precipitation; chromatographic techniques such as HPLC, FPLC, ion exchange  
chromatography, molecular sieve chromatography; size separation methods such as gel  
electrophoresis. Other separation and purification methods known in the art may be used  
as well.

In addition the invention also provides methods for simultaneously characterizing a set of SAGE tag fragments comprising: a) obtaining a RNA sample; b) generating cDNA fragments using a 3' anchored oligo dT primer for first strand synthesis; c) digesting the cDNA generated in step b) with an enzyme; d) isolating 3' cDNA fragments of the digested cDNA; e) amplifying the 3' cDNA fragments of step d) by (i) ligating a SAGE linker to the 3' cDNA; (ii) mixing the 3' cDNA with a sense primer comprising the sequence of the SAGE linker, an antisense primer comprising the sequence of the primer used in step b) or a fragment thereof, and a polymerase enzyme under conditions suitable for amplification; f) purifying the amplified 3' cDNA fragments obtained in step e); g) performing a second amplification comprising generation of longer cDNA fragments from SAGE tags in a multi-well format by mixing said 3' cDNA fragments with a sense primer comprising a SAGE tag sequence and a restriction enzyme sequence, an antisense primer comprising the sequence of the primer used in step b) or a fragment thereof; and a polymerase enzyme under conditions suitable for amplification; and h) cloning and sequencing the products generated in step g).

The 3' anchored oligo dT primer for first strand synthesis can be further attached to an affinity label such as biotin. This allows for isolation of the cDNA or fragments thereof by an affinity-based isolating method using for example streptavidin to recognize and bind the biotin. However, as will be recognized by the skilled artisan, one is not restricted to the use of streptavidin and biotin and any affinity label system may be used, for example, any antigen and its corresponding antibody, *etc.*

The enzyme used to digest the cDNA generated in step c) can be a restriction enzyme for example *NlaIII*. In a preferred embodiment the polymerase enzyme used in steps e) and g) of the method is PLATINUM Taq which provides high specificity and increases yield of the final product.

The steps of cloning and sequencing are well known to the skilled artisan and generically comprise : a) precipitating and purifying the amplified products of step g) in

the multi-well format; b) cloning the purified products into a vector, c) transforming competent bacteria with cloned products; d) screening for transformants; and e) sequencing DNA from transformants to identify the gene encoded by the SAGE tag. In specific embodiments, the positive transformants are screened by direct colony-PCR™ amplifications.

In preferred embodiments of this method more than one SAGE tags are simultaneously identified. This multiple identification provides for high-throughput. The high-throughput generation of longer SAGE tags for gene identification (GLGI) procedure has several important features, for example, (i) 3' cDNAs instead of full-length cDNAs are used as the templates for GLGI amplification. This prevents artificial amplification from non-specific annealing of sense primer. The 3' cDNAs can be amplified to provide sufficient templates for GLGI amplification; (ii) a single antisense primer (in one example the primer is: 5'-ACTATCTAGAGCGGCCGCTT-3' (see also Example 3) is used for all GLGI reactions instead of using combination of the five anchored oligo dT primers. The sequence of the antisense primer is located in 3' end of all the cDNA templates incorporated from anchored oligo dT primers used for the first strand cDNA synthesis. Use of a single primer also increases the efficiency of GLGI amplification significantly as any annealing of this primer with 3' end sequence results in extension during PCR. This feature is particularly useful to amplify the templates with low copies; (iii) Use of PLATINUM Taq polymerase instead of Pfu DNA polymerase increases the yield of final products, while maintaining high specificity; (iv) the GLGI amplified DNAs are directly precipitated and cloned into vector without gel purification, which further prevents loss of amplified products. The inventors contemplate that this is especially important for products with short sizes and for products generated from templates with low copies. Thus, the methods of this invention provide the ability for large-scale identification of expressed genes. Genes of any eukaryotic origin, including human genes may therefore be identified at an accelerated rate by the simple, efficient and low-cost methods set forth herein.

Using the standard convention, "a" or "an" is defined herein to mean one or more than one. Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred  
5      embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

10      The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

15      **FIG. 1. Schematic for GLGI.** (FIG.1A). In this process, first strand cDNA synthesized by oligo-dT is used for PCR. In the first cycle, the template with the SAGE tag binding site is annealed by the sense primer and extended to the end of the template. In the second cycle, extension only occurs from the anchored oligo-dT primer annealed  
20      and paired correctly at the beginning of poly-dA sequences. Exponential amplification only occurs for the template with the SAGE tag binding site. (FIG.1B). GLGI results in the conversion of a 10 bases of SAGE tag to hundred bases of 3' cDNA fragment.

25      **FIG. 2. Size distribution of *Nla*III digested cDNA.** Double strand cDNA was digested by *Nla*III and electrophoresed on a 1.5% agarose gel to demonstrate the size distribution of the digested fragments.

30      **FIG. 3. Specific amplification of 3' sequences corresponding to a specific SAGE tag sequence by GLGI.** In the PCR reaction, each SAGE tag sequence was used as the sense primer, each single dA, dG or dC or a mixture of three anchored oligo-dT primers



was used as the antisense primers. The 3'-end nucleotide for Hs.184776 is dT, for Hs.3463 is dC, and for Hs.118786 is dG.

5 **FIG. 4.** Comparison between RAST-PCR method and GLGI method. A set of 4 SAGE tags was chosen for the analysis. The same RNA from human colon and sense primers were used for both methods. The conditions used for RAST-PCR followed the procedures described in reference (Van den Berg *et al.*, 1999).

10 **FIG.5.** Schematic for high-throughput GLGI.

**FIG.6.** Schematic for high-throughput GLGI amplification.

15 **FIG.7.** Identification of correct 3' sequences for multiple matched SAGE tags. SAGE tags with multiple matches were selected from the high abundant, intermediate abundant and low abundant copies, and those tags were used as the sense primer for GLGI amplification. Gel demonstration of the 3' cDNAs amplified through GLGI.

## **DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS**

### 20 **A. The Present Invention**

The inventors have developed a technique called the Generation of Longer cDNA fragments from SAGE tags for Gene Identification (GLGI), which converts SAGE tags, which are about 10 base pairs in length, into their corresponding 3' cDNA fragments covering hundred bases. The sense primer used comprises about 10 bases corresponding to a SAGE Tag and the antisense primer comprises a single base anchored to an oligo-dT primer. The single base may be dA, dG, or dC. PCR amplification using the primers described above generates a cDNA fragment extending from the SAGE Tag toward the 3' end of the corresponding sequence.

30 Application of the GLGI technique solves two critical issues in the application of the SAGE technique: (i) longer fragments corresponding to novel SAGE tags can be

generated for further studies; and (ii) distinct fragments corresponding to a single SAGE tags can be identified and distinguished. Thus, the development of the GLGI method provides several potential applications. First, it provides a strategy for even wider application of the SAGE technique for quantitative analysis of global gene expression. 5 Second, it can be used to identify the 3' cDNA sequence from any exon within a gene. These exons include ones predicted by bioinformatic tools. Third, a combined application of SAGE/GLGI can be used to complete the catalogue of the expressed genes in human and in other eukaryotic species. And fourth, a combined application of SAGE/GLGI can be applied to define the 3' boundary of expressed genes in the genomic 10 sequences in human and in other eukaryotic genomes.

In the present invention the GLGI technique is further developed herein to identify genes encoding isolated proteins. Isolated proteins are digested by methods known to one of ordinary skill in the art. The protein fragments are then used to obtain 15 nucleotide-sequences-encoding them. These relatively small nucleotide sequences are then used in GLGI wherein a DNA amplification reaction is performed using these nucleotide sequences as sense primers and using a single-base anchored poly-dT sequence as an anti-sense primer. This allows the amplification of DNA towards the 3' end of the gene encoding the isolated protein. Thus, the combination of GLGI with 20 peptide/protein sequencing provides a novel method for gene identification starting with an isolated protein.

The GLGI method is still further developed herein into a high-throughput method for simultaneously converting a large set of SAGE tags into their 3' cDNAs thereby 25 simultaneously characterizing a set of SAGE tag fragments. The method provides for generation of cDNA fragments using a 3' anchored oligo dT primer for first strand synthesis from a RNA sample, digesting this cDNA with an enzyme and isolating and amplifying 3' cDNA fragments. Re-amplifying the 3'cDNA fragments in a multi-well format by GLGI amplification generates longer cDNA fragments corresponding to 30 multiple SAGE tags. Cloning and sequencing then allows identification of the gene. This procedure is simple, rapid, efficient and low-cost and therefore provides a tool for

large-scale identification of expressed genes. Thus, genes of eukaryotic origin, such as human genes may be identified at an accelerated rate.

#### **B. Serial Analysis of Gene Expression (SAGE)**

5 The method for serial analysis of gene expression is described in U.S. Patent 5,866,330 to Kinzler *et al.*, which is incorporated herein by reference. The method involves the identification of a short nucleotide sequence tag at a defined position in a messenger RNA. The tag is used to identify the corresponding transcript and gene from which it was transcribed. By utilizing concatenated tags a rapid quantitative and  
10 qualitative analysis of expressed genes is possible. SAGE is thus useful as a gene discovery tool for the identification of known genes and novel sequence tags corresponding to novel transcripts and genes.

#### **C. Oligonucleotide Probes and Primers**

15 The present invention, in various aspects, will involve the use of nucleic acid hybridization. Hybridization occurs between nucleic acids that have a given degree of "complementarity." Nucleic acid sequences that are "complementary" are those that are capable of base-pairing according to the standard Watson-Crick complementary rules. As used herein, the term "complementary sequences" means nucleic acid sequences that are  
20 substantially identical, or as defined as being capable of annealing to a target nucleic acid segment being described under relatively stringent conditions such as those described herein.

The term primer, as defined herein, is meant to encompass any nucleic acid that is  
25 capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty-five base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded or single-stranded form, although the single-stranded form is preferred. Probes are defined differently, although they may act as primers. Probes, while perhaps capable  
30 of priming, are designed to binding to the target DNA or RNA and need not be used in an amplification process.

Primers should be of sufficient length to provide specific annealing to a RNA or DNA tissue sample. The use of a primer of between about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 20-25, 25-30, 30-35 and 35-40 nucleotides in length allows the formation of a duplex molecule that is both stable and selective. Of particular importance are SAGE derived primers which range from about 10 to 30 bases.

As a general rule, shorter oligomers are easier to make. However, numerous other factors are involved in determining usefulness. Both binding affinity and sequence specificity of an oligonucleotide to its complementary target increases with increasing length. It is contemplated that exemplary oligonucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more base pairs will be used, although others are contemplated. Longer polynucleotides encoding 250, 300, 500, 600, 700, 800, and longer are contemplated as well. Accordingly, nucleotide sequences may be selected for their ability to selectively form duplex molecules with complementary stretches of genes or RNAs or to provide primers for amplification of DNA or RNA from cells, cell lysates and tissues. The method of using probes and primers of the present invention is in the selective amplification and detection of genes, changes in gene expression, changes in mRNA expression wherein one could be detecting virtually any gene or genes of interest from any species. The target polynucleotide will be RNA molecules, mRNA, cDNA or amplified DNA. By varying the stringency of annealing, and the region of the primer, different degrees of homology may be discovered.

Primers may be chemically synthesized by methods well known within the art. Chemical synthesis methods allow for the placement of detectable labels such as fluorescent labels, radioactive labels, *etc.*, to be placed virtually anywhere within the polynucleotide acid sequence. Solid phase method of synthesis also may be used.

The amplification primers may be attached to a solid-phase, for example, a latex bead, a magnetic bead, or the surface of a chip. Thus, the amplification carried out using these primers will be on a solid support/surface.

Furthermore, some primers of the present invention may have a recognition moiety attached. A wide variety of appropriate recognition means are known in the art, including fluorescent labels, radioactive labels, mass labels, affinity labels, chromophores, dyes, electroluminescence, chemiluminescence, enzymatic tags, or other  
5 ligands, such as avidin/biotin, or antibodies, which are capable of being detected and are described below.

### 1. Primer Design

10 According to the present invention, there are disclosed, in one aspect, oligo-dT primers for use in reverse transcription and amplification reactions. These primers are single-base 3'-anchored, *i.e.*, contain a bases at their 3' ends. These bases are the singlets A, G or C. This creates a set of three primers.

15 The particular length of the primer is not believed to be critical, with the dT sequence ranging from about 10 to about 25 bases, with 11 being a preferred embodiment. In some embodiments, the primers are labeled with radioactive species (<sup>32</sup>P, <sup>14</sup>C, <sup>35</sup>S, <sup>3</sup>H, or other isotope), with a fluorophore (rhodamine, fluorescein, GFP) or a chemiluminescent label (luciferase).

20 Yet another primer specific to this invention is the sense prime that is comprised of a SAGE tag sequence. A discussion of these primers is provided U.S. Patent 5,866,330 to Kinzler *et al.*, which is incorporated herein by reference. Other exon-specific or gene-specific primers may be used for the sequencing and characterizing of amplified  
25 sequences.

### 2. Probes

In various contexts, it may be useful to use oligo- or polynucleotides as probes for complementary or hybridizing DNA or RNA molecules. In this regard, one may include  
30 particular "target" sequences in the oligos of the present invention in order to detect the

products by probe hybridization. Alternatively, the probes may recognize unique sequences in the amplified regions upstream of the anchored oligo-dT primers.

### 3. Primer Synthesis

5 Oligonucleotide synthesis is performed according to standard methods. See, for example, Itakura and Riggs (1980). Additionally, U.S. Patent 4,704,362; U. S. Patent 5,221,619; U. S. Patent 5,583,013 each describe various methods of preparing synthetic structural genes.

10 Oligonucleotide synthesis is well known to those of skill in the art. Various different mechanisms of oligonucleotide synthesis have been disclosed in for example, U.S. Patents 4,659,774, 4,816,571, 5,141,813, 5,264,566, 4,959,463, 5,428,148, 5,554,744, 5,574,146, 5,602,244, each of which is incorporated herein by reference. Basically, chemical synthesis can be achieved by the diester method, the triester method,  
15 polynucleotides-phosphorylase-method and by solid-phase chemistry. These methods are discussed in further detail below.

**Diester method.** The diester method was the first to be developed to a usable state, primarily by Khorana and co-workers (Khorana, 1979). The basic step is the  
20 joining of two suitably protected deoxynucleotides to form a dideoxynucleotide containing a phosphodiester bond. The diester method is well established and has been used to synthesize DNA molecules (Khorana, 1979).

**Triester method.** The main difference between the diester and triester methods  
25 is the presence in the latter of an extra protecting group on the phosphate atoms of the reactants and products (Itakura *et al.*, 1975). The phosphate protecting group is usually a chlorophenyl group, which renders the nucleotides and polynucleotide intermediates soluble in organic solvents. Therefore purification's are done in chloroform solutions. Other improvements in the method include (i) the block coupling of trimers and larger  
30 oligomers, (ii) the extensive use of high-performance liquid chromatography for the purification of both intermediate and final products, and (iii) solid-phase synthesis.

**Polynucleotide phosphorylase method.** This is an enzymatic method of DNA synthesis that can be used to synthesize many useful oligodeoxynucleotides (Gillam *et al.*, 1978; Gillam *et al.*, 1979). Under controlled conditions, polynucleotide phosphorylase adds predominantly a single nucleotide to a short oligodeoxynucleotide. Chromatographic purification allows the desired single adduct to be obtained. At least a trimer is required to start the procedure, and this primer must be obtained by some other method. The polynucleotide phosphorylase method works and has the advantage that the procedures involved are familiar to most biochemists.

**Solid-phase methods.** Drawing on the technology developed for the solid-phase synthesis of polypeptides, it has been possible to attach the initial nucleotide to solid support material and proceed with the stepwise addition of nucleotides. All mixing and washing steps are simplified, and the procedure becomes amenable to automation. These syntheses are now routinely carried out using automatic DNA synthesizers.

Phosphoramidite chemistry (Beaucage and Lyer, 1992) has become by far the most widely used coupling chemistry for the synthesis of oligonucleotides. As is well known to those skilled in the art, phosphoramidite synthesis of oligonucleotides involves activation of nucleoside phosphoramidite monomer precursors by reaction with an activating agent to form activated intermediates, followed by sequential addition of the activated intermediates to the growing oligonucleotide chain (generally anchored at one end to a suitable solid support) to form the oligonucleotide product.

#### **D. Amplification**

**PCR™** In some embodiments, poly-A mRNA is isolated and reverse transcribed (referred to as RT) to obtain cDNA which is then used as a template for polymerase chain reaction (referred to as PCR™) based amplification. In other embodiments, cDNA may be obtained and used as a template for the PCR™ reaction. In PCR™, pairs of primers that selectively hybridize to nucleic acids are used under conditions that permit selective hybridization. The term primer, as used herein, encompasses any nucleic acid that is

capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Primers may be provided in double-stranded or single-stranded form, although the single-stranded form is preferred.

5           The primers are used in any one of a number of template dependent processes to amplify the target-gene sequences present in a given template sample. One of the best known amplification methods is PCR<sup>TM</sup> which is described in detail in U.S. Patents 4,683,195, 4,683,202 and 4,800,159, each incorporated herein by reference.

10           In PCR<sup>TM</sup>, two primer sequences are prepared which are complementary to regions on opposite complementary strands of the target-gene(s) sequence. The primers will hybridize to form a nucleic-acid:primer complex if the target-gene(s) sequence is present in a sample. An excess of deoxynucleoside triphosphates are added to a reaction mixture along with a DNA polymerase, *e.g.*, *Taq* polymerase, that facilitates  
15   template-dependent nucleic-acid-synthesis.

          If the target-gene(s) sequence:primer complex has been formed, the polymerase will cause the primers to be extended along the target-gene(s) sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the  
20   extended primers will dissociate from the target-gene(s) to form reaction products, excess primers will bind to the target-gene(s) and to the reaction products and the process is repeated. These multiple rounds of amplification, referred to as "cycles," are conducted until a sufficient amount of amplification product is produced.

25           Next, the amplification product is detected. In certain applications, the detection may be performed by visual means. Alternatively, the detection may involve indirect identification of the product *via* fluorescent labels, chemiluminescence, radioactive scintigraphy of incorporated radiolabel or incorporation of labeled nucleotides, mass labels or even *via* a system using electrical or thermal impulse signals (Affymax  
30   technology).



A reverse transcriptase PCR™ amplification procedure may be performed in order to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook *et al.*, 1989. Alternative methods for reverse transcription utilize thermostable DNA polymerases. These methods are described in WO 90/07641, filed December 21, 1990.

#### E. Hybridization

Hybridization is the technique used to identify nucleic acid products by the nature of the complementarity of a target gene to the hybridization probe or primer. Varying degrees of probe/primer selectivity towards target sequence can be measured.

For applications requiring high selectivity, one typically will employ relatively stringent conditions to form the hybrids, *e.g.*, one will select relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.10 M NaCl at temperatures of about 50°C to about 70°C. Such high stringency conditions tolerate little, if any, mismatch between the probe and the template or target strand, and would be particularly suitable for detecting specific genes or specific mRNA transcripts. It is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide.

For certain applications, it is appreciated that lower stringency conditions are required. Under these conditions, hybridization may occur even though the sequences of probe/primer and target strand are not perfectly complementary, but are mismatched at one or more positions. Conditions may be rendered less stringent by increasing salt concentration and decreasing temperature. For example, a medium stringency condition could be provided by about 0.1 to 0.25 M NaCl at temperatures of about 37°C to about 55°C, while a low stringency condition could be provided by about 0.15 M to about 0.9 M salt, at temperatures ranging from about 20°C to about 55°C. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

In other embodiments, hybridization may be achieved under conditions of, for example, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, at temperatures between approximately 20°C to about 37°C. Other hybridization conditions utilized could include approximately 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 μM MgCl<sub>2</sub>, at temperatures ranging from approximately 40°C to about 72°C.

The selected conditions will depend on the particular circumstances based on the particular criteria required (depending, for example, on the G+C content, type of target nucleic acid, source of nucleic acid, size of hybridization probe, *etc.*). Following washing of the hybridized surface to remove non-specifically bound probe/primer molecules, hybridization is detected, or even quantified, by means of the label.

In general, it is envisioned that hybridization with respect to the primers described herein or in the context of probes will be useful both in solution hybridization, as in PCR<sup>TM</sup>, for the priming of amplification reactions and for the detection of target or reference gene expression, as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) can be adsorbed or otherwise affixed (for example, by affinity separation methods) to a selected matrix or surface. This fixed, single-stranded nucleic acid can then be subject to hybridization with selected probes or primers under desired conditions. Alternatively, the probe or primer may be fixed to the selected matrix or surface for gene detection. Suitable surfaces include chips, latex beads or plates.

#### **F. cDNA Synthesis**

In a preferred embodiment of the invention, the assay is employed for analyzing gene expression patterns using RNA as the starting template. The RNA template may be presented as either total cellular RNA or isolated mRNA. Both types of sample yield comparable results. In still further embodiments, other types of nucleic acids may serve as template in the assay, including genomic or extragenomic DNA, viral RNA or DNA, or nucleic acid polymers generated by non-replicative or artificial means.

In a preferred embodiment of the invention, RNA is converted to cDNA using a oligo-dT primer. Methods of reverse transcribing RNA into cDNA are well known, and described in Sambrook *et al.*, 1989. Alternative methods for reverse transcription utilize thermostable DNA polymerases. These methods are described in WO90/07641. In  
5 alternative embodiments, avian myeloblastosis virus reverse transcriptase (AMV-RT), or Maloney murine leukemia virus reverse transcriptase (MoMLV-RT) may be used. Other enzymes are contemplated as well.

In another embodiment, RNA targets may be reverse transcribed using other  
10 non-specific primers, such as an anchored oligo-dT primer, or random sequence primers. An advantage of this embodiment is that the "unfractionated" quality of the mRNA sample is maintained because the sites of priming are non-specific, *i.e.*, the products of this RT reaction will serve as template for any desired target in the subsequent PCR™ amplification. This allows samples to be archived in the form of DNA, which is more  
15 stable than RNA.

### G. Sequencing

Methods for sequencing are well known in the art, in particular, the chain-termination technique pioneered by Sanger *et al.* in the mid-1970's. Recent  
20 developments have increased dramatically the number of bases that can be sequenced in a short period of time. The following U.S. patents, dealing with DNA sequencing, are incorporated by reference: U.S. Patents 6,004,446; 5,985,556; 5,968,743; 5,876,934; 5,866,328; 5,858,671; 5,846,727; 5,821,060; 5,821,058; 5,817,797; 5,780,232; 5,755,943; 5,674,716; 5,639,608; 5,608,063; 5,523,206; 5,455,008; 5,432,065; 5,405,746; 5,360,523;  
25 5,308,751; and 5,207,880.

### H. Restriction Enzymes

Restriction-enzymes recognize specific short DNA sequences four to eight nucleotides long (see Table 1), and cleave the DNA at a site within this sequence.  
30 Restriction enzymes are used to cleave cDNA molecules at sites corresponding to various restriction-enzyme recognition sites. In context of this invention, the enzyme NlaIII is

often used in the SAGE technique and the SAGE tags often are comprised of NlaIII recognition sequences. The sense primers in the present invention may further comprise a restriction enzyme recognition sequence, such as the BamHI sequence, to allow easier cloning amplified DNA fragments for further analysis.

5

As the sequence of the recognition site is known (see list below), primers can be designed comprising nucleotides corresponding to the recognition sequences. If the primer sets have in addition to the restriction recognition sequence, degenerate sequences corresponding to different combinations of nucleotide sequences, one can use the amplified cDNA fragments that have the particular restriction enzyme sequence for cloning the cDNA into cloning vectors. The list below exemplifies the currently known restriction enzymes that may be used in the invention.

10

**Table 1: Restriction Enzymes**

15

Enzyme Name	Recognition Sequence
AatII	GACGTC
Acc65 I	GGTACC
Acc I	GTMKAC
Aci I	CCGC
Acl I	AACGTT
Afe I	AGCGCT
Afl II	CTTAAG
Afl III	ACRYGT
Age I	ACCGGT
Ahd I	GACNNNNNGTC
Alu I	AGCT
Alw I	GGATC
AlwN I	CAGNNNCTG
Apa I	GGGCCC
ApaL I	GTGCAC
Apo I	RAATTY
Asc I	GGCGCGCC
Ase I	ATTAAT
Ava I	CYCGRG
Ava II	GGWCC
Avr II	CCTAGG
Bae I	NACNNNNGTAPyCN
BamH I	GGATCC
Ban I	GGYRCC

Ban II	GRGCYC
Bbs I	GAAGAC
Bbv I	GCAGC
BbvC I	CCTCAGC
Bcg I	CGANNNNNNTGC
BciV I	GTATCC
Bcl I	TGATCA
Bfa I	CTAG
Bgl I	GCCNNNNNGGC
Bgl II	AGATCT
Blp I	GCTNAGC
Bmr I	ACTGGG
Bpm I	CTGGAG
BsaA I	YACGTR
BsaB I	GATNNNNATC
BsaH I	GRCGYC
Bsa I	GGTCTC
BsaJ I	CCNNGG
BsaW I	WCCGGW
BseR I	GAGGAG
Bsg I	GTGCAG
BsiE I	CGRYCG
BsiHKA I	GWGCWC
BsiW I	CGTACG
Bsl I	CCNNNNNNNNGG
BsmA I	GTCTC
BsmB I	CGTCTC
BsmF I	GGGAC
Bsm I	GAATGC
BsoB I	CYCGRG
Bsp1286 I	GDGCHC
BspD I	ATCGAT
BspE I	TCCGGA
BspH I	TCATGA
BspM I	ACCTGC
BsrB I	CCGCTC
BsrD I	GCAATG
BsrF I	RCCGGY
BsrG I	TGTACA
Bsr I	ACTGG
BssH II	GCGCGC
BssK I	CCNNGG
Bst4C I	ACNGT
BssS I	CACGAG
BstAP I	GCANNNNNNTGC
BstB I	TTCGAA

BstE II	GGTNACC
BstF5 I	GGATGNN
BstN I	CCWGG
BstU I	CGCG
BstX I	CCANNNNNNTGG
BstY I	RGATCY
BstZ17 I	GTATAC
Bsu36 I	CCTNAGG
Btg I	CCPuPyGG
Btr I	CACGTG
Cac8 I	GCNNGC
Cla I	ATCGAT
Dde I	CTNAG
Dpn I	GATC
Dpn II	GATC
Dra I	TTTAAA
Dra III	CACNNNGTG
Drd I	GACNNNNNNGTC
Eae I	YGGCCR
Eag I	CGGCCG
Ear I	CTCTTC
Eci I	GGCGGA
EcoN I	CCTNNNNNAGG
EcoO109 I	RGGNCCY
EcoR I	GAATTC
EcoR V	GATATC
Fau I	CCCGCNNNN
Fnu4H I	GCNGC
Fok I	GGATG
Fse I	GGCCGGCC
Fsp I	TGCGCA
Hae II	RGCGCY
Hae III	GGCC
Hga I	GACGC
Hha I	GCGC
Hinc II	GTYRAC
Hind III	AAGCTT
Hinf I	GANTC
HinP1 I	GCGC
Hpa I	GTAAAC
Hpa II	CCGG
Hph I	GGTGA
Kas I	GGCGCC
Kpn I	GGTACC
Mbo I	GATC
Mbo II	GAAGA

Mfe I	CAATTG
Mlu I	ACGCGT
Mly I	GAGTCNNNNN
Mnl I	CCTC
Msc I	TGGCCA
Mse I	TTAA
Msl I	CAYNNNNRTG
MspA1 I	CMGCKG
Msp I	CCGG
Mwo I	GCNNNNNNNGC
Nae I	GCCGGC
Nar I	GGCGCC
Nci I	CCSGG
Nco I	CCATGG
Nde I	CATATG
NgoMI V	GCCGGC
Nhe I	GCTAGC
Nla III	CATG
Nla IV	GGNNCC
Not I	GCGGCCGC
Nru I	TCGCGA
Nsi I	ATGCAT
Nsp I	RCATGY
Pac I	TTAATTAA
PaeR7 I	CTCGAG
Pci I	ACATGT
PflF I	GACNNNGTC
PflM I	CCANNNNNTGG
PleI	GAGTC
Pme I	GTTTAAAC
Pml I	CACGTG
PpuM I	RGGWCCY
PshA I	GACNNNGTC
Psi I	TTATAA
PspG I	CCWGG
PspOM I	GGGCCC
Pst I	CTGCAG
Pvu I	CGATCG
Pvu II	CAGCTG
Rsa I	GTAC
Rsr II	CGGWCCG
Sac I	GAGCTC
Sac II	CCGCGG
Sal I	GTCGAC
Sap I	GCTCTTC
Sau3A I	GATC

Sau96 I	GGNCC
Sbf I	CCTGCAGG
Sca I	AGTACT
ScrF I	CCNGG
SexA I	ACCWGGT
SfaN I	GCATC
Sfc I	CTRYAG
Sfi I	GGCCNNNNNGGCC
Sfo I	GGCGCC
SgrA I	CRCCGGYG
Sma I	CCCGGG
Sml I	CTYRAG
SnaB I	TACGTA
Spe I	ACTAGT
Sph I	GCATGC
Ssp I	AATATT
Stu I	AGGCCT
Sty I	CCWWGG
Swa I	ATTTAAAT
Taq I	TCGA
Tfi I	GAWTC
Tli I	CTCGAG
Tse I	GCWGC
Tsp45 I	GTSAC
Tsp509 I	AATT
TspR I	CAGTG
Tth111 I	GACNNNGTC
Xba I	TCTAGA
Xcm I	CCANNNNNNNNTGG
Xho I	CTCGAG
Xma I	CCCGGG
Xmn I	GAANNNTTC

## I. Polymerases

### 1. Reverse Transcriptases

According to the present invention, a variety of different reverse transcriptases  
5 may be utilized. The following are representative examples.

**M-MLV Reverse Transcriptase.** M-MLV (Moloney Murine Leukemia Virus  
Reverse Transcriptase) is an RNA-dependent DNA polymerase requiring a DNA primer  
and an RNA template to synthesize a complementary DNA strand. The enzyme is a  
10 product of the *pol* gene of M-MLV and consists of a single subunit with a molecular



weight of 71kDa. M-MLV RT has a weaker intrinsic RNase H activity than Avian Myeloblastosis Virus (AMV) reverse transcriptase which is important for achieving long full-length complementary DNA (>7 kB).

5 M-MLV can be use for first strand cDNA synthesis and primer extensions. Storage recommend at -20°C in 20 mM Tris-HCl (pH 7.5), 0.2M NaCl, 0.1 mM EDTA, 1 mM DTT, 0.01% Nonidet® P-40, 50% glycerol. The standard reaction conditions are 50 mM Tris-HCl (pH 8.3), 7 mM MgCl<sub>2</sub>, 40 mM KCl, 10 mM DTT, 0.1 mg/ml BSA, 0.5 mM <sup>3</sup>H-dTTP, 0.025 mM oligo(dT)<sub>50</sub>, 0.25 mM poly(A)<sub>400</sub> at 37°C.

10

**M-MLV Reverse Transcriptase, RNase H Minus.** This is a form of Moloney murine leukemia virus reverse transcriptase (RNA-dependent DNA polymerase) which has been genetically altered to remove the associated ribonuclease H activity (Tanese and Goff, 1988). It can be used for first strand cDNA synthesis and primer extension.

15 Storage is at 20°C in 20 mM Tris-HCl (pH 7.5), 0.2M NaCl, 0.1 mM EDTA, 1 mM DTT, 0.01% Nonidet® P-40, 50% glycerol.

**AMV Reverse Transcriptase.** Avian Myeloblastosis Virus reverse transcriptase is a RNA dependent DNA polymerase that uses single-stranded RNA or DNA as a  
20 template to synthesize the complementary DNA strand (Houts *et al.*, 1979). It has activity at high temperature (42°C - 50°C). This polymerase has been used to synthesize long cDNA molecules.

Reaction conditions are 50 mM Tris-HCl (pH 8.3), 20 mM KCl, 10 mM MgCl<sub>2</sub>,  
25 500 µM of each dNTP, 5 mM dithiothreitol, 200 µg/ml oligo-dT<sub>(12-18)</sub>, 250 µg/ml polyadenylated RNA, 6.0 pMol <sup>32</sup>P-dCTP, and 30 U enzyme in a 7 µl volume. Incubate 45 min at 42°C. Storage buffer is 200 mM KPO<sub>4</sub> (pH 7.4), 2 mM dithiothreitol, 0.2% Triton X-100, and 50% glycerol. AMV may be used for first strand cDNA synthesis, RNA or DNA dideoxy chain termination sequencing, and fill-ins or other DNA  
30 polymerization reactions for which Klenow polymerase is not satisfactory (Maniatis *et al.*, 1976).

## 2. DNA polymerases

The present invention also contemplates the use of various DNA polymerase. Exemplary polymerases are described below.

5

***Bst* DNA Polymerase, Large Fragment.** *Bst* DNA Polymerase Large Fragment is the portion of the *Bacillus stearothermophilus* DNA Polymerase protein that contains the 5'→3' polymerase activity, but lacks the 5'→3' exonuclease domain. *BST* Polymerase Large Fragment is prepared from an *E. coli* strain containing a genetic fusion of the *Bacillus stearothermophilus* DNA Polymerase gene, lacking the 5'→3' exonuclease domain, and the gene coding for *E. coli* maltose binding protein (MBP). The fusion protein is purified to near homogeneity and the MBP portion is cleaved off *in vitro*. The remaining polymerase is purified free of MBP (Iiyy *et al.*, 1991).

15

*Bst* DNA polymerase can be used in DNA sequencing through high GC regions (Hugh & Griffin, 1994; McClary *et al.*, 1991) and Rapid Sequencing from nanogram amounts of DNA template (Mead *et al.*, 1991). The reaction buffer is 1X ThermoPol Buffer (20 mM Tris-HCl (pH 8.8 at 25°C), 10 mM KCl, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 mM MgSO<sub>4</sub>, 0.1% Triton X-100). Supplied with enzyme as a 10X concentrated stock.

20

*Bst* DNA Polymerase does not exhibit 3'→5' exonuclease activity. 100 µ/ml BSA or 0.1% Triton X-100 is required for long term storage. Reaction temperatures above 70°C are not recommended. Heat inactivated by incubation at 80°C for 10 min. *Bst* DNA Polymerase cannot be used for thermal cycle sequencing. Unit assay conditions are 50 mM KCl, 20 mM Tris-HCl (pH 8.8), 10 mM MgCl<sub>2</sub>, 30 nM M13mp18 ssDNA, 70 nM M13 sequencing primer (-47) 24 mer (NEB #1224), 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 100 µM <sup>3</sup>H-dTTP, 100 µg/ml BSA and enzyme. Incubate at 65°C. Storage buffer is 50 mM KCl, 10 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 0.1 mM EDTA, 0.1% Triton-X-100 and 50% glycerol. Storage is at -20°C.

30

**VENTR<sup>®</sup> DNA Polymerase and VENTR<sup>®</sup> (exo<sup>-</sup>) DNA Polymerase.** Vent<sub>R</sub> DNA Polymerase is a high-fidelity thermophilic DNA polymerase. The fidelity of Vent<sub>R</sub> DNA Polymerase is 5-15-fold higher than that observed for Taq DNA Polymerase (Mattila *et al.*, 1991; Eckert and Kunkel, 1991). This high fidelity derives in part from an integral 3'→5' proofreading exonuclease activity in Vent<sub>R</sub> DNA Polymerase (Mattila *et al.*, 1991; Kong *et al.*, 1993). Greater than 90% of the polymerase activity remains following a 1 h incubation at 95°C.

Vent<sub>R</sub> (exo<sup>-</sup>) DNA Polymerase has been genetically engineered to eliminate the 3'→5' proofreading exonuclease activity associated with Vent<sub>R</sub> DNA Polymerase (Kong *et al.*, 1993). This is the preferred form for high-temperature dideoxy sequencing reactions and for high yield primer extension reactions. The fidelity of polymerization by this form is reduced to a level about 2-fold higher than that of Taq DNA Polymerase (Mattila *et al.*, 1991; Eckert & Kunkel, 1991). Vent<sub>R</sub> (exo<sup>-</sup>) DNA Polymerase is an excellent choice for DNA sequencing and is included in Circum-Vent-Sequencing-Kit (see pages 118 and 121).

Both Vent<sub>R</sub> and Vent<sub>R</sub> (exo<sup>-</sup>) are purified from strains of *E. coli* that carry the Vent DNA Polymerase gene from the archaea *Thermococcus litoralis* (Perler *et al.*, 1992). The native organism is capable of growth at up to 98°C and was isolated from a submarine thermal vent (Belkin and Jannasch, 1985). They are useful in primer extension, thermal cycle sequencing and high temperature dideoxy-sequencing.

**DEEP VENTR<sup>™</sup> DNA Polymerase and DEEP VENTR<sup>™</sup> (exo<sup>-</sup>) DNA Polymerase.** Deep Vent<sub>R</sub> DNA Polymerase is the second high-fidelity thermophilic DNA polymerase available from New England Biolabs. The fidelity of Deep Vent<sub>R</sub> DNA Polymerase is derived in part from an integral 3'→5' proofreading exonuclease activity. Deep Vent<sub>R</sub> is even more stable than Vent<sub>R</sub> at temperatures of 95 to 100°C (see graph).

Deep Vent<sub>R</sub> (exo<sup>-</sup>) DNA Polymerase has been genetically engineered to eliminate the 3'→5' proofreading exonuclease activity associated with Deep Vent<sub>R</sub> DNA

Polymerase. This exo- version can be used for DNA sequencing but requires different dNTP/ddNTP ratios than those used with Vent<sub>R</sub> (exo-) DNA Polymerase. Both Deep Vent<sub>R</sub> and Deep Vent<sub>R</sub> (exo-) are purified from a strain of *E. coli* that carries the Deep Vent<sub>R</sub> DNA Polymerase gene from *Pyrococcus species* GB-D (Perler *et al.*, 1996). The native organism was isolated from a submarine thermal vent at 2010 meters (Jannasch *et al.*, 1992) and is able to grow at temperatures as high as 104°C. Both enzymes can be used in primer extension, thermal cycle sequencing and high temperature dideoxy-sequencing.

10        **T7 DNA Polymerase (unmodified).** T7 DNA polymerase catalyzes the replication of T7 phage DNA during infection. The protein dimer has two catalytic activities: DNA polymerase activity and strong 3'→5' exonuclease (Hori *et al.*, 1979; Engler *et al.*, 1983; Nordstrom *et al.*, 1981). The high fidelity and rapid extension rate of the enzyme make it particularly useful in copying long stretches of DNA template.

15        T7 DNA Polymerase consists of two subunits: T7 gene 5 protein (84 kilodaltons) and *E. coli* thioredoxin (12 kilodaltons) (Hori *et al.*, 1979; Studier *et al.*, 1990; Grippo & Richardson, 1971; Modrich & Richardson, 1975; Adler & Modrich, 1979). Each protein is cloned and overexpressed in a T7 expression system in *E. coli* (Studier *et al.*, 1990). It  
20        can be used in second strand synthesis in site-directed mutagenesis protocols (Bebenek & Kunkel, 1989).

      The reaction buffer is 1X T7 DNA Polymerase Buffer (20 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol). Supplement with 0.05 mg/ml BSA and dNTPs.  
25        Incubate at 37°C. The high polymerization rate of the enzyme makes long incubations unnecessary. T7 DNA Polymerase is not suitable for DNA sequencing.

      Unit assay conditions are 20 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 0.05 mg/ml BSA, 0.15 mM each dNTP, 0.5 mM heat denatured calf  
30        thymus DNA and enzyme. Storage conditions are 50 mM KPO<sub>4</sub> (pH 7.0), 0.1 mM EDTA, 1 mM dithiothreitol and 50% glycerol. Store at -20°C.

**DNA Polymerase I (*E. coli*).** DNA Polymerase I is a DNA-dependent DNA polymerase with inherent 3'→5' and 5'→3' exonuclease activities (Lehman, 1981). The 5'→3' exonuclease activity removes nucleotides ahead of the growing DNA chain, allowing nick-translation. It is isolated from *E. coli* CM 5199, a lysogen carrying  $\lambda$ *polA* transducing phage (obtained from N.E. Murray) (Murray & Kelley, 1979). The phage in this strain was derived from the original *polA* phage encoding wild-type Polymerase I.

Applications include nick translation of DNA to obtain probes with a high specific activity (Meinkoth and Wahl, 1987) and second strand synthesis of cDNA (Gubler & Hoffmann, 1983; D'Alessio & Gerard, 1988). The reaction buffer is *E. coli* Polymerase I/Klenow Buffer (10 mM Tris-HCl (pH 7.5), 5 mM MgCl<sub>2</sub>, 7.5 mM dithiothreitol). Supplement with dNTPs.

DNase I is not included with this enzyme and must be added for nick translation reactions. Heat inactivation is for 20 min at 75°C. Unit assay conditions are 40 mM KP0<sub>4</sub> (pH 7.5), 6.6 mM MgCl<sub>2</sub>, 1 mM 2-mercaptoethanol, 20 μM dAT copolymer, 33 μM dATP and 33 μM <sup>3</sup>H-dTTP. Storage conditions are 0.1 M KP0<sub>4</sub> (pH 6.5), 1 mM dithiothreitol, and 50% glycerol. Store at -20°C.

**DNA Polymerase I, Large (Klenow) Fragment.** Klenow fragment is a proteolytic product of *E. coli* DNA Polymerase I that retains polymerization and 3'→5' exonuclease activity, but has lost 5'→3' exonuclease activity. Klenow retains the polymerization fidelity of the holoenzyme without degrading 5' termini.

A genetic fusion of the *E. coli polA* gene, that has its 5'→3' exonuclease domain genetically replaced by maltose binding protein (MBP). Klenow Fragment is cleaved from the fusion and purified away from MBP. The resulting Klenow fragment has the identical amino and carboxy termini as the conventionally prepared Klenow fragment.

30

Applications include DNA sequencing by the Sanger dideoxy method (Sanger *et al.*, 1977), fill-in of 3' recessed ends (Sambrook *et al.*, 1989), second-strand cDNA synthesis, random priming labeling and second strand synthesis in mutagenesis protocols (Gubler, 1987).

5

Reactions conditions are 1X *E. coli* Polymerase I/Klenow Buffer (10 mM Tris-HCl (pH 7.5), 5 mM MgCl<sub>2</sub>, 7.5 mM dithiothreitol). Supplement with dNTPs (not included). Klenow fragment is also 50% active in all four standard NEBuffers when supplemented with dNTPs. Heat inactivated by incubating at 75°C for 20 min. Fill-in  
10 conditions: DNA should be dissolved, at a concentration of 50 µg/ml, in one of the four standard NEBuffers (1X) supplemented with 33 µM each dNTP. Add 1 unit Klenow per µg DNA and incubate 15 min at 25°C. Stop reaction by adding EDTA to 10 mM final concentration and heating at 75°C for 10 min. Unit assay conditions 40 mM KP04 (pH 7.5), 6.6 mM MgCl<sub>2</sub>, 1 mM 2-mercaptoethanol, 20 µM dAT copolymer, 33 µM  
15 dATP and 33 µM <sup>3</sup>H-dTTP. Storage conditions are 0.1 M KP04 (pH 6.5), 1 mM dithiothreitol, and 50% glycerol. Store at -20°C.

**Klenow Fragment (3'→5' exo<sup>-</sup>).** Klenow Fragment (3'→5' exo<sup>-</sup>) is a proteolytic product of DNA Polymerase I which retains polymerase activity, but has a mutation  
20 which abolishes the 3'→5' exonuclease activity and has lost the 5'→3' exonuclease (Derbyshire *et al.*, 1988).

A genetic fusion of the *E. coli polA* gene, that has its 3'→5' exonuclease domain genetically altered and 5'→3' exonuclease domain replaced by maltose binding protein  
25 (MBP). Klenow Fragment exo<sup>-</sup> is cleaved from the fusion and purified away from MBP. Applications include random priming labeling, DNA sequence by Sanger dideoxy method (Sanger *et al.*, 1977), second strand cDNA synthesis and second strand synthesis in mutagenesis protocols (Gubler, 1987).

Reaction buffer is 1X *E. coli* Polymerase I/Klenow Buffer (10 mM Tris-HCl (pH 7.5), 5 mM MgCl<sub>2</sub>, 7.5 mM dithiothreitol). Supplement with dNTPs. Klenow Fragment exo- is also 50% active in all four standard NEBuffers when supplemented with dNTPs. Heat inactivated by incubating at 75°C for 20 min. When using Klenow  
5 Fragment (3'→5' exo-) for sequencing DNA using the dideoxy method of Sanger *et al.* (1977), an enzyme concentration of 1 unit/5 µl is recommended.

Unit assay conditions are 40 mM KPO<sub>4</sub> (pH 7.5), 6.6 mM MgCl<sub>2</sub>, 1 mM 2-mercaptoethanol, 20 µM dAT copolymer, 33 µM dATP and 33 µM <sup>3</sup>H-dTTP. Storage  
10 conditions are 0.1 M KPO<sub>4</sub> (pH 7.5), 1 mM dithiothreitol, and 50% glycerol. Store at -20°C.

**T4 DNA Polymerase.** T4 DNA Polymerase catalyzes the synthesis of DNA in the 5'→3' direction and requires the presence of template and primer. This enzyme has a  
15 3'→5' exonuclease activity which is much more active than that found in DNA Polymerase I. Unlike *E. coli* DNA Polymerase I, T4 DNA Polymerase does not have a 5'→3' exonuclease function.

Purified from a strain of *E. coli* that carries a T4 DNA Polymerase overproducing  
20 plasmid. Applications include removing 3' overhangs to form blunt ends (Tabor & Struhl, 1989; Sambrook *et al.*, 1989), 5' overhang fill-in to form blunt ends (Tabor & Struhl, 1989; Sambrook *et al.*, 1989), single strand deletion subcloning (Dale *et al.*, 1985), second strand synthesis in site-directed mutagenesis (Kunkel *et al.*, 1987), and probe labeling using replacement synthesis (Tabor & Struhl, 1989; Sambrook *et al.*,  
25 1989).

The reaction buffer is 1X T4 DNA Polymerase Buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol (pH 7.9 at 25°C)). Supplement with 40 µg/ml BSA and dNTPs (not included in supplied 10X buffer). Incubate at temperature  
30 suggested for specific protocol.

It is recommended to use 100  $\mu$ M of each dNTP, 1-3 units polymerase/ $\mu$ g DNA and incubation at 12°C for 20 min in the above reaction buffer (Tabor & Struhl, 1989; Sambrook *et al.*, 1989). Heat inactivated by incubating at 75°C for 10 min. T4 DNA  
5 Polymerase is active in all four standard NEBuffers when supplemented with dNTPs.

Unit assay conditions are 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol (pH 7.9 at 25°C), 33  $\mu$ M dATP, dCTP and dGTP, 33  $\mu$ M <sup>3</sup>H dTTP, 70  $\mu$ g/ml denatured calf thymus DNA, and 170  $\mu$ g/ml BSA. Note: These are not  
10 suggested reaction conditions; refer to Reaction Buffer. Storage conditions are 100 mM KP0<sub>4</sub> (pH 6.5), 10 mM 2-mercaptoethanol and 50% glycerol. Store at -20°C.

### 3. RNA polymerases

RNA polymerases for use in the present invention are exemplified as follows.

15

#### T7 RNA Polymerase SP6 RNA Polymerase and T3 RNA Polymerase.

Initiation of transcription with T7, SP6 RNA and T3 RNA Polymerase Polymerases is highly specific for the T7 and SP6 phage promoters, respectively. Cloning vectors have been developed which direct transcription from the T7 SP6 or T3 promoter through  
20 polylinker cloning sites (Schenborn & Meirendorf, 1985). These vectors allow *in vitro* synthesis of defined RNA transcripts from a cloned DNA sequence. Under optimal conditions, greater than 700 moles of T7 RNA transcript can be synthesized per mole of DNA template (Noren *et al.*, 1990). RNA produced using the SP6 and T7 RNA polymerases is biologically active as mRNA (Krieg & Melton, 1984) and can be  
25 accurately spliced (Green *et al.*, 1983). Anti-sense RNA, produced by reversing the orientation of the cloned DNA insert, has been shown to specifically block mRNA translation *in vivo* (Melton, 1985).

Labeled single-stranded RNA transcripts of high specific activity are simple to  
30 prepare with T7 and SP6 RNA polymerases (Sambrook *et al.*, 1989). Increased levels of detection in nucleic acid hybridization reactions can also be obtained due to the greater



stability of RNA:DNA hybrids with respect to RNA:RNA or DNA:DNA hybrids (Zinn *et al.*, 1983).

5 SP6 RNA Polymerase is isolated from SP6 phage-infected *Salmonella typhimurium* LT2Z (Butler & Chamberlin, 1982). T7 RNA Polymerase is isolated from *E. coli* BL21 carrying the plasmid pAR1219 which contains T7 *gene 1* under the control of the inducible *lac* UV6 promoter (Davanloo *et al.*, 1984). Applications include preparation of radiolabeled RNA probes (Sambrook *et al.*, 1989), RNA generation for *in vitro* translation (Sambrook *et al.*, 1989), RNA generation for studies of RNA structure, 10 processing and catalysis (Sambrook *et al.*, 1989) and expression control via antisense RNA.

Reaction 1X RNA Polymerase Buffer: (40 mM Tris-HCl (pH 7.9), 6 mM MgCl<sub>2</sub>, 2 mM spermidine, 10 mM dithiothreitol). Supplement with 0.5 mM each ATP, UTP, 15 GTP, CTP (not included) and DNA template containing the appropriate promoter. Incubate at 37°C (T7 RNA polymerase) or 40°C (SP6 RNA polymerase).

Dithiothreitol is required for activity. Both enzymes are extremely sensitive to salt inhibition. For best results overall salt concentration should not exceed 50 mM. SP6 20 RNA polymerase is 30% more active at 40°C than at 37°C. Higher yields of RNA may be obtained by raising NTP concentrations (up to 4 mM each). Mg<sup>2+</sup> concentration should be raised to 4 mM above the total NTP concentration. Additionally, inorganic pyrophosphatase should be added to a final concentration of 4 units/ml. SP6 RNA polymerase is supplied with a control template (NEB#207B). The template is a pSP64 25 vector containing a 1.38 kB insert, linearized at 3 different restriction sites. Transcription with SP6 RNA polymerase results in three runoff fragments of 1.38 kB, 0.55 kB and 0.22 kB.

Storage conditions are 100 mM NaCl, 50 mM Tris-HCl (pH 7.9), 1 mM EDTA, 30 20 mM 2-mercaptoethanol, 0.1% Triton-X-100 and 50% glycerol. Store at -20°C.

T3 RNA polymerase is a DNA dependent RNA polymerase which exhibits extremely high specificity for T3 promoter sequences. The enzyme will incorporate 32P, 35S and 3H-labeled nucleotide triphosphates. It is used in the synthesis of RNA transcripts for hybridization probes in vitro translation, RNase protection assays or RNA processing substrates.

One unit of T3 RNA polymerase is defined as the amount of enzyme required to catalyze the incorporation of 5nmol of CTP into acid insoluble product in 60 minutes at 37°C in a total volume of 100µl. The reaction conditions are as follows, 40mM Tris-HCl (pH 7.9), 6 mM MgCl<sub>2</sub>, 10mM DTT, 10mM NaCl, 2mM spermidine, 0.5% Tween®-20, 0.5mM each ATP, GTP, DTP, and UTP, 0.5µCi [<sup>3</sup>H] CTP, and 2µg supercoiled pSP6/T3 Vector DNA. Promega provide a T3 RNA polymerase extracted from recombinant *E. coli*.

#### 15 J. Analysis of Sequence Data-/Bioinformatics

The sequences generated using GLGI can be used to match gene databases (e.g., GenBank, EMBL, DDBJ, UniGene Human Database). Each sequence will be identified as a known gene, EST sequence, or novel sequences without matches. There are many bioinformatic tools used for gene prediction in genomic DNA, for example, GenScan™ program.

#### 20 K. Protein Purification

In context of the present invention it will be desirable to isolate and purify proteins. Protein purification techniques are well known to those of skill in the art. These techniques involve, at one level, the crude fractionation of the cellular milieu to polypeptide and non-polypeptide fractions. Having separated the polypeptide from other proteins, the polypeptide of interest may be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, exclusion chromatography; polyacrylamide gel

electrophoresis; isoelectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography or even HPLC.

5 Certain aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of an encoded protein or peptide. The term "purified protein or peptide" as used herein, is intended to refer to a composition, isolatable from other components, wherein the protein or peptide is purified to any degree relative to its naturally-obtainable state. A purified protein or peptide therefore also refers to a protein or peptide, free from the environment in which it may naturally occur.

10

Generally, "purified" will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50%,  
15 about 60%, about 70%, about 80%, about 90%, about 95% or more of the proteins in the composition.

Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These  
20 include, for example, determining the specific activity of an active fraction, or assessing the amount of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate  
25 the degree of purity, herein assessed by a "-fold purification number." The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

30 Various techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium

5 sulphate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

10 There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will  
15 ~~generally result in a greater "fold" purification than the same technique utilizing a low~~ pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

20 It is known that the migration of a polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE (Capaldi *et al.*, 1977). It will therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or partially purified expression products may vary.

25 High Performance Liquid Chromatography (HPLC) and FPLC are characterized by a very rapid separation with extraordinary resolution of peaks. This is achieved by the use of very fine particles and high pressure to maintain an adequate flow rate. Separation can be accomplished in a matter of minutes, or at most an hour. Moreover, only a very small volume of the sample is needed because the particles are so small and close-packed  
30 that the void volume is a very small fraction of the bed volume. Also, the concentration

of the sample need not be very great because the bands are so narrow that there is very little dilution of the sample.

5 Gel chromatography, or molecular sieve chromatography, is a special type of partition chromatography that is based on molecular size. The theory behind gel chromatography is that the column, which is prepared with tiny particles of an inert substance that contain small pores, separates larger molecules from smaller molecules as they pass through or around the pores, depending on their size. As long as the material of which the particles are made does not adsorb the molecules, the sole factor determining  
10 rate of flow is the size. Hence, molecules are eluted from the column in decreasing size, so long as the shape is relatively constant. Gel chromatography is unsurpassed for separating molecules of different size because separation is independent of all other factors such as pH, ionic strength, temperature, *etc.* There also is virtually no adsorption, less zone spreading and the elution volume is related in a simple matter to molecular  
15 weight.

Affinity Chromatography is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule that it can specifically bind to. This is a receptor-ligand type interaction. The column material is synthesized by  
20 covalently coupling one of the binding partners to an insoluble matrix. The column material is then able to specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (alter pH, ionic strength, temperature, *etc.*).

25 A particular type of affinity chromatography useful in the purification of carbohydrate containing compounds is lectin affinity chromatography. Lectins are a class of substances that bind to a variety of polysaccharides and glycoproteins. Lectins are usually coupled to agarose by cyanogen bromide. Concanavalin A coupled to Sepharose was the first material of this sort to be used and has been widely used in the isolation of  
30 polysaccharides and glycoproteins other lectins that have been include lentil lectin, wheat germ agglutinin which has been useful in the purification of N-acetyl glucosaminyl

residues and *Helix pomatia* lectin. Lectins themselves are purified using affinity chromatography with carbohydrate ligands. Lactose has been used to purify lectins from castor bean and peanuts; maltose has been useful in extracting lectins from lentils and jack bean; N-acetyl-D galactosamine is used for purifying lectins from soybean; N-acetyl glucosaminyI binds to lectins from wheat germ; D-galactosamine has been used in obtaining lectins from clams and L-fucose will bind to lectins from lotus.

The matrix should be a substance that itself does not adsorb molecules to any significant extent and that has a broad range of chemical, physical and thermal stability. The ligand should be coupled in such a way as to not affect its binding properties. The ligand should also provide relatively tight binding. And it should be possible to elute the substance without destroying the sample or the ligand. One of the most common forms of affinity chromatography is immunoaffinity chromatography. The generation of antibodies that would be suitable for use in accord with the present invention is discussed below.

#### L. Sequencing Proteins

Protein sequencing may be carried out by techniques well known in the art such as those involving the sequential removal of amino acids from one end of the protein and identifying each removed amino acid in turn (Edman's Degradation). Other techniques to obtain amino acid sequence information use mass spectrometry, typically using fast atom bombardment to ionize the sample. In fast atom bombardment, a sample dissolved in a liquid is bombarded with atoms or ions. Charged molecules resulting from this process are directed into the spectrometer and detected. An example of this technique is described in the text entitled "Macro Molecular Sequencing and Synthesis Selected Methods and Applications", 1988, published by Alan R. Liss, Inc., specifically at pages 83 to 99 in an article in such text entitled "Mass Spectrometry in Bio-Pharmaceutical Research" by Steven A. Carr et al. 1988, Several modifications of these techniques are well known to the skilled artisan and any of the techniques used for protein sequencing may be used in context of the present invention.

Typically protein sequencing methods involve digesting the large protein molecule into smaller fragments. These fragments are then separated or purified and then subject to the sequencing method.

## 1. Digesting Proteins

5 Digesting purified and/or isolated protein molecules to obtain smaller fragments can be carried out using proteolytic enzymes, known as proteases, to obtain a variety of N-terminal, C-terminal and internal fragments. Some of the well known proteases include trypsin, chymotrypsin, elastase, collagenase, leupeptin, and endoproteinases.  
10 Other protein digesting enzymes are also present and may be used in this invention and are well known to one of ordinary skill in the art and. Examples of fragments may include contiguous residues of the protein sequence 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, 75, 80, 85, 90, 95, 100, or more amino acids in length.

## 2. Separating Protein Fragments

15 These digested protein fragments may be separated or further purified according to known methods, such as precipitation *e.g.* ammonium sulfate precipitation; HPLC; ion exchange chromatography; affinity chromatography (including immunoaffinity  
20 chromatography); and/or various size separations such as sedimentation, gel electrophoresis (SDS-PAGE), gel filtration or molecular sieve chromatography. All these methods are described above in detail.

High Performance Liquid Chromatography (HPLC) and FPLC are preferred  
25 methods since they provide very rapid separation with extraordinary resolution of peaks. Separation can be accomplished in a matter of minutes, or at most an hour and furthermore only a very small volume of the sample is needed. Also, the concentration of the sample need not be very great because the bands are so narrow that there is very little dilution of the sample. This is ideal for digested protein fragments.

30

## M. Obtaining Nucleic Acid Sequences from Protein Sequences

The protein fragment sequences obtained above can then be used to obtain nucleic acid sequences by techniques well known to one of skill in the art. The techniques include artificial synthesis of nucleic acid polymers. Table 2 below describes the degeneracy of codons and provides the corresponding amino acid sequences. As known to the skilled artisan, one can use the codon preference or bias of an organism if known.

**TABLE 2**

Amino Acids			Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

10

The nucleotides generated in the present invention include those encoding the isolated and purified proteins fragments as described above. It will also be understood that nucleic acid sequences (and their encoded amino acid sequences) may include additional residues, such as additional 5' or 3' sequences.

15



## N. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

### EXAMPLE 1

#### Materials and Methods

**SAGE Tags.** A group of SAGE tags 10 bases long were selected from the SAGE tag-sequences-database-generated-from-epithelium cells of normal colon (Zhang *et al.*, 1997) (<http://www.ncbi.nlm.nih.gov/SAGE/sagerec.cgi?rec=166>). Each selected SAGE tag sequence was searched in the UniGene database (<http://www.ncbi.nlm.nih.gov/SAGE/SAGetag.cgi?tag>) to identify it as a matched or an unmatched tag sequence. Each matched sequence was given the appropriate Unigene ID number. Both matched and unmatched tags were used in the experiments.

**RNA samples and cDNA synthesis.** The same RNA sample from epithelium cells of normal human colon tissue was used for this experiment (Zhang *et al.*, 1997). RNA samples from 24 different human tissues were also used for the detection of multiple expression (CloneTech). First strand cDNAs were generated through oligo-dT priming with a cDNA synthesis kit (Life Technologies), following the manufacturer's instruction. After cDNA synthesis, the excess free oligo-dT primers were removed using a MicroSpin S-300 column (Amersham Pharmacia).

**PCR conditions.** *Pfu* DNA polymerase (Stratagene) was used with 10x buffer (200 mM Tris-HCl pH 8.8, 100 mM KCl, 100 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 mM MgSO<sub>4</sub>, 1%

Triton X-100, 1 mg/ml BSA). Two mM MgCl<sub>2</sub> was added in each reaction to increase the Mg<sup>++</sup> concentration. The PCR mixture contained 1 x buffer, 2 mM MgCl<sub>2</sub>, 0.3 mM dNTPs, 0.04 unit/μl *Pfu* polymerase, 3 ng/μl sense primer, 1.5 ng/μl anchored oligo-dT primer (single or mixture) in final volume of 20 or 50 μl. The PCR reactions were performed first at 94°C 1 min, followed by 5 cycles at 94°C 20 sec, 50 to 53°C 20 sec, 72°C 20 sec. The conditions were then changed to 25 cycles at 94°C 20 sec, 60°C 20 sec, and 72°C 20 sec. The reactions were kept at 72°C for five minutes for the last cycle.

**DNA cloning and sequencing.** PCR amplified fragments were cloned into pCR-Blunt vector (InvitroGen). Positive clones were screened using PCR with M13 reverse and M13 forward (-20) primers located in the vector, or using *EcoRI* digestion. Plasmids were prepared with a plasmid purification kit (Qiagen). Sequencing reactions were performed with PE big-dye kit (PE Applied Biosystems) with M13 reverse primer, following the manufacturer's instruction.

**Database search.** All the sequences generated from the clones were searched using the BLAST program for alignment (<http://www.ncbi.nlm.nih.gov/BLAST/>).

## EXAMPLE 2

### Results and Discussion

The inventors envisioned that the amplification of a particular template corresponding to a particular SAGE tag will proceed as depicted in the schematic in FIG. 1, using a combination of a sense primer containing a SAGE tag sequence and a single-base anchored oligo-dT antisense primer. In this process, only the cDNA templates containing the binding sequences for the SAGE tag will be annealed and extended in the first PCR cycle. In the second cycle, the extension will only happen from that single-base anchored oligo-dT primer which anneals at the 5' end of the poly-dA sequences with the anchored-nucleotide correctly paired to the last nucleotide before the poly-dA sequence. Extension of all other anchored primers annealed along the poly-dA sequences

will be blocked because of presence of the anchor nucleotide. The resulting extended templates will exclude poly-dA/dT sequences. Only the cDNA templates containing the SAGE tag sequence will undergo exponential amplification in the following PCR cycles. Thus, only copies of the same size will be generated.

5

The expected size distribution of amplified sequences using this strategy should be up to several hundred bases, because of the use of *Nla*III digestion in the SAGE process for SAGE tag collection (Velculescu *et al.*, 1995). *Nla*III is a restriction enzyme recognizing CATG. As shown in FIG. 2, the size distribution of *Nla*III digested cDNA was centered between 200 to 500 base pairs.

10

**Design of primer.** Each SAGE tag contains only a 10 base sequence. To increase the length of the primers for efficient PCR priming, CATG, a *Nla*III recognition site used for collecting SAGE Tag fragments (Velculescu *et al.*, 1995), was added 5' of the SAGE tag. A *Bam*HI recognition site, GGATCC, was added 5' of the primer to increase the primer size and to provide a potential site for subcloning. For the anchored oligo-dT primers, a single-base anchor dA, dG, or dC was attached to the 3' end of the oligo-dT primer (Khan *et al.*, 1991; Kiriangkum *et al.*, 1992; Liang and Pardee, 1992, Liang *et al.*, 1994; Wang and Rowley, 1998). To determine the best length of oligo-dT sequences, different numbers of dT nucleotides from 11 to 20 were tested, with dT11 giving the best results.

15

20

**Optimizing PCR condition.** Various PCR conditions were tested in order to maximize the specificity and efficiency of amplification. In the PCR reaction, the anchored primers were either combined separately with each sense primer, or a mixture of equal amounts of dA, dG and dC anchored primers was used with the sense primer. *Pfu* DNA polymerase was chosen for the PCR amplification because it showed greater fidelity of amplification compared with regular Taq DNA polymerase (Lundberg *et al.*, 1991) (data not show). The  $Mg^{++}$  concentration played an important role in determining the specificity and the yield of the PCR products. Satisfactory results were usually obtained at the final concentration of 4 mM  $Mg^{++}$ . The number of PCR cycles is

30

important to maintain the specificity of the amplification. Over-amplification with a high number of PCR cycles could result in non-specific amplification.

**Amplification of longer sequences from SAGE tags.** A group of SAGE tags generated from colon tissues was selected for the analysis (Zhang *et al.*, 1997) (Table 3). PCR<sup>TM</sup> was performed with each sense primer containing the SAGE tag sequence and individual or mixed anchored oligo-dT primers, combined with cDNAs from colon tissue generated by oligo-dT priming. The PCR products were electrophoresed through an agarose gel, and cloned into vector for sequencing analysis. FIG. 3 shows examples of the PCR amplification with three SAGE tags that matched to known sequences. The last nucleotide before the poly-dA sequences for those three sequences (Hs.184776, Hs.3463 and Hs.118786) is dT, dC, and dG respectively. The inventors obtained the expected results. The amplification occurred only in the reaction with dA, dG and dC anchored oligo-dT for these three sequences. When the dA, dG and dC anchored oligo-dT primers were mixed for each reaction, the same amplification products can be generated even though the amplification efficiency was lower due to the competition of binding between these three primers. These data indicate that the reaction can be simplified into a single reaction using a combination of dA, dG and dC anchored oligo-dT primers. Table 3 summarizes the results generated from these experiments. For the matched SAGE tag sequences, amplification occurred when the correct anchor primers were used except for Hs.194659, which was amplified by dG anchored oligo-dT but the matched sequences ended with dT. The size distribution of these amplified fragments ranged from 77 to 382 base pairs. cDNA fragments were also generated from three unmatched SAGE tags, and they represent novel sequences.

25

**Identify the correct sequence from multiple sequences that matched with the same SAGE Tag.** When matching SAGE tag sequences in databases, a single SAGE tag may align with several sequences. For example, nine out of 40 SAGE tag sequences show matches to multiple Unigene Clusters (Zhang *et al.*, 1997). Other than sharing the same SAGE tag sequence, these matched sequences have no homology and are derived from various different tissues. To test this issue experimentally, 12 SAGE tags were used

30

for amplification with cDNA samples from 24 different human tissues. Four out of these 12 tags generated multiple templates. For example, the SAGE tag (GTCATCACCA) generated five different sequences from five different tissues (fetal liver, skeletal muscle, spinal cord, trachea and colon), and two different sequences from the same tissue (spinal cord) (Table 4). All of these fragments contained the same SAGE tag sequence, but the rest of the sequences showed no homology. Among these sequences, the ones from colon tissue all matched the previous amplified sequences in the colon (Table 3). These data indicate that a SAGE tag itself may not be sufficient to serve as a unique identifier for a particular sequence, when several sequences share the same SAGE tag sequences. It is important to distinguish which one of the matched sequences is the correct sequence corresponding to the particular SAGE tag. To avoid the uncertainty when different sequences are expressed from different tissues, it will be necessary to generate the fragment from the same tissue used to generate the SAGE tag. The inventors' observations also indicate that relying only on a database search to identify the sequence corresponding to a SAGE tag may provide misleading information. Direct amplification of the specific template with the inventors strategy will be very useful for confirmation of the validity of a particular SAGE tag.

**Table 3. Summary of GLGI results from SAGE Tags**

SAGE Tags (10 base)	Unigene ID	3' end nucleotide in matched sequences*	Amplified by anchored oligo dT	Length of sequen ce (bs)	Match to original sequence**
GGAAGGTTTA	Hs.105484	dT/dG	dT	77	+
AGATCCCAAG	Hs.50813	dC/dG	dC	84	+
CTTATGGTCC	Hs.179608	dT	dT	86	+
AGGATGGTCC	Hs.71779	dC	dC	112	+
GTCATCACCA	Hs.32966	dC	dC	119	+
GACCAGTGGC	Hs.143131	dC/dT	dC	135	+
CTGTTGGTGA	Hs.3463	dC	dC	148	+
ACTGGGTCTA	Hs.227823	dG	dG	150	+
TACGGTGTGG	Hs.105460	dC	dC	166	+
CGGTGGGACC	Hs.99175	dC/dT/dG	dC	200	+
CCTTCAAATC	Hs.23118	dC/dT	dC	220	+
GGAGGCGCTC	Hs.33455	dT/dG	dT	238	+
AAGAAGATAG	Hs.73848	dT	dT	317	+
GATCCCAACT	Hs.118786	dG/dT/dC	dG	329	+
GAACAGCTCA	Hs.194659	dT	dG	382	+
AGGTGACTGG	-	-	dC	156	-
CACCTAGTTG	-	-	dT	170	-
CCTGTCTGCC	-	-	dT	249	-

\*The 3' end nucleotides from all the sequences were included in each matched Unigene cluster.

\*\*The amplified sequences were matched to databases again. The last three sequences have no matches and represent novel sequences.

During the course of the research, the inventors became aware of a report describing a method RAST-PCR (Rapid RT-PCR Analysis of Unknown SAGE Tags) for analyzing unknown SAGE Tags (van den Berg *et al.*, 1999). The authors used a sense primer that was designed based on a SAGE tag. However, the antisense primer was the M13 sequence tailed to 5' oligo-dT<sub>24</sub> used for cDNA synthesis. In the process of cDNA synthesis, oligo-dT primers anneal randomly along the poly-A sequences in the mRNA template. The resulting cDNAs include various lengths of poly-dA/dT sequences at the 3' of the cDNA, even from the same mRNA template. Using the M13 sequence tailed to the oligo-dT as the antisense primer for PCR will generate multiple fragments with different sizes or a smear due to the inclusion of different length of poly-dA sequences. Using the conditions described in that paper (Van den Berg, 1999), the inventors obtained the results the inventors expected, namely smears (FIG. 4).

**Table 4. Detection of heterogeneous sequences in various tissues containing the same SAGE Tag**

SAGE TAG	Positive tissues	Unigene ID	length of sequence
CGGTGGGACC	Colon, Thymus, Small intestine	Hs.99175	200
	Small intestine	no match	368
	Thymus	no match	90
AGATCCCAAG	Colon, Heart, Placenta, Thymus	Hs.50813	84
	Placenta	no match	53
	Skeletal muscle	Hs.85937	282
	Testis	no match	227
	Thymus, Placenta	no match	51
CTTATGGTCC	Bone marrow	Hs.237416	393
	Bone marrow	no match	144
	Colon	Hs.179608	86
GTCATCACCA	Fetal liver, Spinal cord	Hs.222346	125
	Skeletal muscle	Hs.1288	399
	Spinal cord	Hs.9641	394
	Trachea	no match	225
	colon	Hs.32966	136

The development of the GLGI method provides several potential applications. First, it provides a strategy for even wider application of the SAGE technique for quantitative analysis of global gene expression. Second, it can be used to identify the 3' cDNA sequence from any exon within a gene. These exons can include the ones predicted by bioinformatic tools. Third, a combined application of SAGE/GLGI can be applied to define the 3' boundary of expressed genes in the genomic sequences in human and in other eukaryotic genomes.

### EXAMPLE 3

#### High-throughput GLGI

A high-throughput GLGI procedure is also developed by the present inventors for converting a large set of SAGE tag sequences into gene identities.

**Materials and Methods.** SAGE tags were selected from the SAGE tag sequences generated from human and mouse myeloid cells, including 203 SAGE tags with multiple matches and 89 SAGE tags without matches. A set of 20 SAGE tags with a single match was used as controls to demonstrate the specificity of GLGI amplification.

5

The same RNA samples from human and mouse myeloid cells used for SAGE analysis were used as the templates for GLGI amplification. mRNAs from 5 µg of total RNA of each sample were isolated with Oligo (dT)<sub>25</sub> Dynabeads (Dyna), following the manufacturer's protocol. Poly(dA/dT) cDNAs were synthesized using a cDNA synthesis kit (Cat. No: 18267-021, Life Technologies) and the 5' biotinylated, 3' anchored oligo (dT) primers were used for first strand cDNA synthesis (5' biotin-ATCTAGAGCGGCCGC-T16-A,G, CA,CG and CC) (Wang *et al.*, 2000). The double-strand cDNAs were then digested with *NlaIII*, and 3' cDNAs were isolated with streptavidin beads (Dyna), following the manufactures protocol. In order to generate enough 3' cDNAs for GLGI analysis, 3'-cDNA-templates-were-amplified by PCR as the following: SAGE linker A or B was ligated to the 3' cDNAs bound to the beads (Linker A: 5'- TTTGGATTTGCTGGTGCAGTACAAGCTTAATAGGGACATG - 3' and 5'- pTCCCTATTAAGCCTAGTTGTACTGCACCAGCAAATCC [amino mod. C7]- 3'; or Linker B: 5'- TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGA CATG - 3' and 5'- pTCCCCGTACATCGTTAGAAGCTTGAATTCGAGCAG [amino mod. C7]- 3')( [http://www.sagenet.org/sage\\_protocol.htm](http://www.sagenet.org/sage_protocol.htm)). The ligated 3' cDNAs were then amplified by 20 cycles of PCR at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with PLATINUM Taq polymerase (Life Technologies), SAGE sense primer (5'- GGATTTGCTGGTGCAG TACA - 3' for linker A; or 5'- CTGCTCGAATTCAAGCTTCT - 3' for linker B)( [http://www.sagenet.org/sage\\_protocol.htm](http://www.sagenet.org/sage_protocol.htm)) and antisense primer (5' - ACTATCTAGAGCGGCCGCTT- 3') located in the 5' end of anchored oligo dT primers used for the first strand cDNA synthesis. The amplified templates were extracted by phenol/chloroform, precipitated by ethanol/NH<sub>4</sub>OAc/glycogen, and resuspended in TE buffer for GLGI amplification.



The sense primer used for GLGI amplification included 14 bases (CATG + 10 base SAGE tag sequence) at the 3' end and 6 bases (GGATCC, BamH I sites) at the 5' of the primer, giving a total of 20 bases for each primer: 5'-GGATCCCATGXXXXXXXXXX -3' (Chen *et al.*, 2000). Sense primers were synthesized in 96 well format and the concentration was adjusted to 50ng/μl with TE. GLGI master mixtures were prepared for each reaction, containing 1x PCR buffer (20 mM TrisCl pH 8.4, 50 mM KCl), 2 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 1.5 units / 0.3 μl PLATINUM Taq polymerase, 60 ng / 1.2 μl antisense primer (5'-ACTATCTAGAGCGGCCGCTT-3'), and 0.5 - 5 ng of 3' cDNAs. The reaction mixtures were aliquoted into a 96-well plate at 28.8 μl per well. Sense primers (60 ng / 1.2 μl) were then added into each well. GLGI reactions were performed in PE GeneAmp PCR Systems 9600 or 9700. The conditions used were 94°C for 2 min, followed by five cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The conditions were then changed to 20-25 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Reactions were kept at 72°C for 5 min for the last cycle. The amplified products were directly precipitated in the 96-well PCR plate by adding 100μl of precipitation mixture to each well, containing 1μl of glycogen (20 mg/ml, Roche), 15μl of 7.5M NH<sub>4</sub>OAc and 84μl of 100% ethanol. The plate was sealed with Tape pads (QIAGEN, Inc), vortexed, and kept at room temperature for 15 min. After spinning at 4000 rpm for 35 min at 4°C (SORVALL RC5C plus; rotor: SH3000), the supernatants were removed, 150μl of 70% ethanol were added per well to wash the DNA, and the plate were spun at 4000 rpm for 15 minutes. The supernatants were removed again, the pallets were air-dried, and dissolved in 5μl of dH<sub>2</sub>O. Two μl of DNA, 0.7 μl of salt solution, 0.7 μl of water, and 6 ng of pCR4-TOPO vector were used for each ligation reaction with TOPO TA cloning kit for sequencing (Invitrogen). The ligation reactions were performed at room temperature for 25 min. For transformation, 2 μl of ligation were mixed with 50 μl of TOPO10 competent cells (Invitrogen), kept on ice for 20 min, then heated at 42°C for 30 s, and moved on ice. SOC media (250 μl) were added per well. Plate was sealed, shaken at 37°C for 60 min at 225 rpm. The transformants were spread on LB plates containing 50 ng/ml

of kanamycin and grew over night at 37°C. Positive clones were screened by direct colony-PCR. PCR master mixtures were prepared, containing 1x PCR buffer (10 mM TrisCl pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>), 0.1 mM dNTPs, 0.5 units / 0.1 µl Taq polymerase (TaKaRa), 60 ng of sense primer (M13 reverse primer) and 60 ng of antisense primer (M13 forward (-20) primer). The reaction mixtures were aliquoted into a 96-well plate at 25µl per well, and colonies were picked into the reaction mixtures with sterile pipette tips. PCR was performed in PE GeneAmp PCR Systems 9600 or 9700. The conditions used were 94°C for 2 min, followed by 25 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s. The reactions were kept at 72°C for 5 min after the last cycle. 75µl of precipitation mixture were added per well to precipitate DNAs, containing 22 µl of dH<sub>2</sub>O, 15µl of 2M NaClO<sub>4</sub> and 38 µl of 2-propanol. The plate was sealed, vortexed, and kept at room temperature for 5 min. After spinning at 4000 rpm for 35 min at 4°C, the supernatants were removed, 150µl of 70% ethanol were added per well to wash the DNA, and the plate were spun at 4000 rpm for 25 minutes. Supernatants were removed again, the pallets were air-dried, and dissolved in 10µl of dH<sub>2</sub>O. Sequencing mixtures were prepared in a total volume of 7µl , containing 0.8µl of big-dye pre-mixture, 1.4µl of dilution buffer (400 mM TrisCl pH 9.0, 10 mM MgCl<sub>2</sub>), 30 ng / 0.3 µl of sequence primer (M13 reverse primer or M13 forward (-20) primer), 1.5µl H<sub>2</sub>O, and 3µl of DNA templates. Sequencing reactions were performed at 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min for 99 cycles. The final sequencing products were precipitated by adding 75µl of precipitation mixture, consisting of 64µl of 100% ethanol/3M NaOAc mixture (25:1), 1µl of glycogen (20 mg/ml) and 10µl dH<sub>2</sub>O. The plate was sealed, vortexed, and kept at room temperature for 15 min. After spinning at 4000 rpm for 35 min at 4°C, the supernatants were removed, 150µl of 70% ethanol were added per well to wash the DNA, and the plate were spun at 4000 rpm for 15 minutes. The supernatants were removed, the pallets were air-dried, and dissolved in 3µl of loading dye. One µl was loaded in 5% sequencing gels. Four to six clones were sequenced for higher abundant SAGE tags, and 8 to 12 clones were sequenced for low abundant SAGE tags. Sequences were collected with an ABI 377 sequencer.

All collected sequences were matched to GenBank Database (NR and ESTs, <http://www.ncbi.nlm.nih.gov/BLAST/>) through BLAST. Any mismatch between the SAGE tag sequence used for GLGI amplification and the SAGE tag sequence of the matched sequence in database was considered as non-specific amplification, and these sequences were eliminated from further analysis. The matched sequence ID was used to search UniGene database to obtain the UniGene cluster ID.

**Results and Discussion.** The details of the high-throughput GLGI method are outlined in FIG.5 and FIG. 6. Double-strand poly(dA/dT) cDNAs are synthesized and digested with NlaIII. The 3' fragments are recovered with streptavidin-coated beads. Large quantity of 3' cDNAs templates can be generated by PCR amplifications of 3' cDNAs. GLGI amplification are performed. Then, 3' cDNA fragments corresponding to each specific SAGE tag are generated, cloned and sequenced. All the procedures are designed in 96 format to facilitate large-scale analyses. All the reagents used herein are optimized to guarantee the result and minimize expenses.

The high-throughput GLGI procedure has several differences as compared to the GLGI, for example, (i) 3' cDNAs instead of full-length cDNAs are used as the templates for GLGI amplification. This prevents artificial amplification from non-specific annealing of sense primer to sequences upstream of the last CATG. The 3' cDNAs can be amplified to provide sufficient templates for GLGI amplification; (ii) a single antisense primer (5'-ACTATCTAGAGCGGCCGCTT-3') is used for all GLGI reactions instead of using combination of the five anchored oligo dT primers. The sequence of the antisense primer is located in 3' end of all the cDNA templates incorporated from anchored oligo dT primers used for the first strand cDNA synthesis. The inventors have observed that the anchored oligo dT primers are unstable which can hinder the successful performance of GLGI. Use of the single primer also increased the efficiency of GLGI amplification significantly as any annealing of this primer with 3' end sequence results in extension during PCR. In contrast, the use of five anchored oligo dT primers results in extension by PCR only when correctly paired primers anneal. This feature is particularly useful to amplify the templates with low copies; (iii) PLATINUM Taq polymerase

instead of Pfu DNA polymerase was used for GLGI amplification, in order to increase the yield of final products, while maintaining high specificity; (iv) the GLGI amplified DNAs were directly precipitated and cloned into vector without gel purification, to prevent the loss of amplified products. This is contemplated to be particularly important for products with short sizes and for products generated from templates with low copies. The inventors' data showed that these changes significantly increase efficiency and specificity for GLGI amplification of 3' cDNAs, especially for templates expressed at low level.

The SAGE tags selected for the analysis herein include SAGE tags with single match, SAGE tags with multiple matches and SAGE tags without matches. FIG. 7 shows an example of the PCR amplifications. Table 5 summarizes these results. Nineteen out of 20 single-matched SAGE tags in the control reactions were converted into single 3' cDNA sequences and matched to the original matched single UniGene clusters. Seventy nine out of 89 unmatched novel SAGE tags were converted into longer 3' cDNA sequences proved by the presence of 3' poly dA/dT tail, no CATG site within the sequences, and no matches to known sequences. One hundred and eighty out of 203 of GLGI reactions from multiple matched SAGE tags generated 3' sequences, most of which (>90%), matched to a single UniGene cluster among the original multiple matched UniGene clusters. The efficiency for detection is parallel with the abundance of the SAGE tags. For higher abundant templates, the rate of success was nearly 100 percent. For the templates with low copies, the efficiency of detection was lower than that for high abundant SAGE tags. The inventors contemplate that this effect can be caused by low levels of template which reaches the limitation of the amplification.

**Table 5. Summary of GLGI results.**

Number of Copy	SAGE tags	Number of matched UniGene clusters	GLGI identified genes
Over 50	6	Single match	6
	150	Multiple match	136
	3	No match	3
49 to 2	9	Single match	9
	37	Multiple match	34
	74	No match	68
1	5	Single match	4
	16	Multiple match	10
	12	No match	8
Total	312		278

Thus, the high-throughput GLGI procedure provides high efficiency for large-scale gene identification based on SAGE Tag sequences. By using this procedure, hundreds of interesting SAGE tags can be simultaneously converted into their 3' cDNA fragments. A large number of genes from genomes are expressed at low level, and these expressed genes can only be detected by SAGE technique. The combination of this GLGI procedure with large sets of SAGE tags detected from low copy templates provides an efficient way to identify these genes. Thus, this procedure will accelerate the completion of identification of expressed genes in the human genome as well as in other eukaryotic genomes.

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both

chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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